Domain architecture of the smooth-muscle plasma membrane: regulation by annexins

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Individual signalling events are processed in distinct, spatially segregated domains of the plasma membrane. In a smooth muscle, the sarcolemma is divided into domains of focal adhesions alternating with caveolae-rich zones, both harbouring a specific subset of membrane-associated proteins. Recently, we have demonstrated that the sarcolemmal lipids are similarly segregated into domains of cholesterol-rich lipid rafts and glycerophospholipid-rich non-raft regions. In the present study, we provide a detailed structural analysis of the relationship between these proteinaceous and lipid domains. We demonstrate that the segregation of plasmalemmal protein constituents is intimately linked to that of the membrane lipids. Our results imply that lipid segregation is critical for the preservation of membrane protein architecture and essential for directional translocation of proteins to the sarcolemma. We show that the membrane lipid segregation is supported by the annexin protein family in a Ca^{2+}-dependent manner. Eukaryotic cells harbour numerous, tissue-specific subsets of annexins. By examining the significance of this variety in a smooth muscle, we demonstrate that four different annexins target membrane sites of distinct lipid composition and that each annexin requires a different $[Ca^{2+}]$ for its translocation to the sarcolemma. Our results suggest that the interactions of annexins with distinct plasma membrane regions promote membrane segregation and, in combination with their individual Ca^{2+} sensitivity, might allow a spatially confined, graded response to a multitude of extra- or intracellular stimuli.

Key words: annexin, Ca^{2+}, lipid microdomain, membrane segregation, sarcolemma, smooth muscle.

INTRODUCTION

Undergoing constant changes in length and charged with the efficient transmission of force, smooth muscle cells need to process a multitude of regulatory signals simultaneously (see [1,2] for reviews). These challenging functional demands are reflected in the exceptionally complex and precise structural organization of their plasma membrane [3,4]. Through their intracellular connection with the actin cytoskeleton and their transmembrane linkage to extracellular components [5], adherens junctions transmit contractile tension to the surrounding connective tissue and alternate with flexible 'hinge' domains containing numerous caveolae [6].

The smooth-muscle plasma membrane is further subdivided into domains of 'liquid-ordered' membrane rafts, which are rich in cholesterol and in lipids with saturated acyl chains, and into less-ordered and more fluid non-raft regions, enriched in glycerophospholipids with polyunsaturated acyl chains [7–11]. Although significant progress has been made in the biochemical and biophysical characterization of raft structure, molecular composition and dynamics, their physiological importance is not fully understood [12,13]. In a smooth muscle, in particular, it is not clear if the well-documented segregation of membrane-associated proteins, localized either in adherens junctions or caveolae, is accompanied by the corresponding segregation of membrane lipids.

In the present study, we show that selective extraction of membrane lipids leads to redistribution of the specific associated proteins. Extraction of cholesterol leads to a relocation of caveolin, however, it has no effect on the periodic spacing of the focal adhesions delineated by vinculin. In contrast, extraction of glycerophospholipids results in the solubilization of vinculin, whereas caveolin remains associated with detergent-resistant cholesterol-rich membranes.

Smooth-muscle-cell contraction is regulated by changes in the intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$). Hence the well-ordered sarcolemmal structure of contractile cells provides a unique opportunity to investigate the plasmalemmal domain organization and its regulation in response to fluctuations in $[Ca^{2+}]_i$. By focusing our investigation on the ubiquitously expressed annexins, a family of Ca^{2+}-dependent, membrane-associated proteins, we show that each annexin requires a subtly different individual level of free calcium for its translocation to the plasma membrane. Moreover, individual annexins associate with membrane domains of distinct lipid composition. Selective extraction of cholesterol prevents the relocation of annexin 6, but not of annexin 4 to the sarcolemma. In contrast, extraction of glycerophospholipids results in solubilization of annexins 1 and 4, whereas annexins 2 and 6 remain associated with detergent-resistant membranes.

Since annexins are present in virtually all eukaryotic cells [14] our findings point to a general principle of membrane domain organization and its rearrangement in response to fluctuations in intracellular calcium and apply to signalling pathways in general.

EXPERIMENTAL

Antibodies

Antibody specificity for immunohistochemistry necessitated the use of human tissue. Consent was obtained from the Medical Ethical Commission of the Canton of Bern. Monoclonal antibodies against annexins 1, 2, 4 and 6 and a polyclonal antibody against caveolin were purchased from Transduction Laboratories (Lexington, KY, U.S.A.). Monoclonal antibodies against RhoA,

Abbreviations used: DRM, detergent-resistant membrane; MCD, methyl-β-cyclodextrin; PE, phosphatidylethanolamine.

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vinculin and transferrin receptor were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), Sigma and Zymed (San Francisco, CA, U.S.A.) respectively.

Immunohistochemistry and electron microscopy

Musculus detrusor vesicae was obtained during routine urinary bladder surgery and processed for contraction/relaxation experiments, electron microscopy and ultrathin cryosectioning as described previously [11]. Cholesterol was extracted (60 min, 30 °C) by incubation of tissue strips in Tyrodes' solution containing 2 % MCD (methyl-β-cyclodextrin; Sigma). Immunolabelling was performed as described by Jostarndt-Fögen et al. [15] and fluorescent labelling using Cy3-conjugated (Jackson, Baltimore, MD, U.S.A.) or Alexa-conjugated (Molecular Probes, Eugene, OR, U.S.A.) secondary antibodies. Negative controls were generated by applying a non-binding primary antibody. Immunostained ultrathin cryo sections were viewed in a Zeiss Axiophot fluorescent microscope and images collected with a digital charge-coupled-device Ultrapix Slowscan camera (Astrocam; Gloor Instruments, Uster, Switzerland). Electron micrographs were viewed in a Siemens 400 electron microscope.

Isolation of smooth-muscle microsomes: sucrose-gradient ultracentrifugation

Unless otherwise stated, all procedures were performed at 4 °C or on ice. Smooth-muscle microsomal membranes were isolated from porcine stomach smooth muscle by a method described earlier [11]. Minced muscle (100 g) was routinely extracted in 300 ml of buffer A (60 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$ and 20 mM imidazol, pH 7.0). After low-speed centrifugation at 10 000 ***g*** for 30 min, the supernatant was filtered through glass wool and then subjected to high-speed centrifugation at 50 000 ***g*** for 90 min. The pellets obtained thereby were washed three times (with intervening centrifugations at 10 000 ***g*** for 30 min) in 10 vol. of buffer B (120 mM KCl, 0.2 mM $CaCl_2$ and 20 mM imidazol, pH 7.0) and finally resuspended in 10 ml of the same buffer.

Smooth-muscle microsomes (1 ml; 10 mg/ml of total protein) were diluted 2-fold in buffer B containing 80 % (w/v) sucrose and 2 % Triton X-100, incubated at 4 °C for 30 min and overlaid by a discontinuous gradient of 30 % (10 ml)/5 % (5 ml) sucrose containing 1 % Triton X-100 prepared using buffer B. In a separate set of experiments, 0.5 ml of microsomes (10 mg/ml of total protein) were diluted 2-fold in buffer B containing 80 % sucrose and various concentrations of Triton X-100, and overlaid by a discontinuous gradient of 30 % (3.5 ml)/5 % (0.5 ml) sucrose in buffer B. When low $[Ca^{2+}]$ was required, 2 mM EGTA was added to the solution. The gradients were subjected to ultracentrifugation at 4 °C (16 h, 100 000 ***g***) in a swing-out rotor. Fractions of 1 ml or 0.5 ml were collected starting from the top of a centrifugation tube. TLC, Western blotting, SDS/PAGE and data analysis were performed as described previously [11,16].

Extraction of microsomal membranes with Triton X-100 and Ca^{2+} sensitivity

Aliquots (100 μl) of the microsomal membrane preparations (protein concentration, 5 mg/ml) were incubated at 20 °C for 30 min in buffer B containing the indicated concentrations of Triton X-100 and/or additional $CaCl_2$ and EGTA to obtain the $[Ca^{2+}]_{free}$ as indicated in the Figure legends. The suspensions were subjected to a low-speed centrifugation (10 000 ***g***, 30 min). Each of the pellets was resuspended in 100 μl of buffer B containing the corresponding [Triton X-100] or $[Ca^{2+}]_{free}$ and again subjected to low-speed centrifugation. The resulting pellets, made up to 100 μl, were analysed by Western blotting.

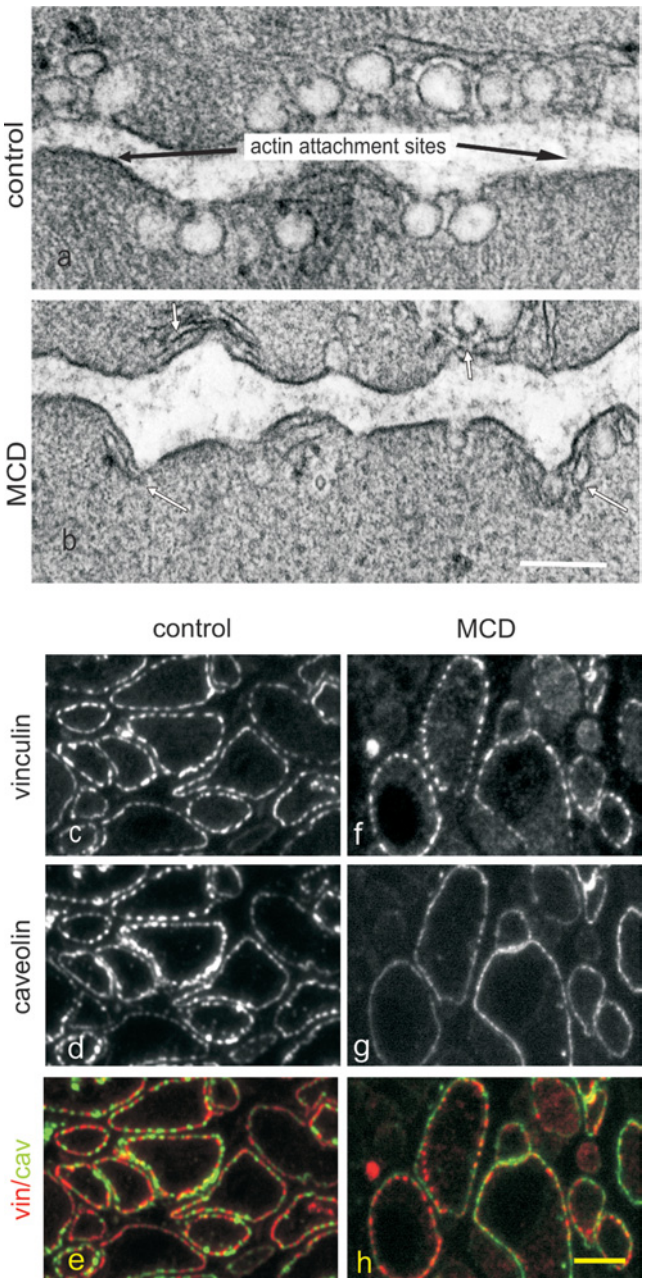

Figure 1 Extraction of cholesterol affects plasma membrane macrodomain structure

(**a**, **b**) Ultrathin transverse sections of a smooth-muscle bundle before (**a**) and after (**b**) treatment with MCD. (**a**) Actin attachment sites (filled arrows) alternate with caveolar regions. (**b**) The depletion of cholesterol leads to a flattening of caveolae, however, without compromising sarcolemmal integrity or that of the sarcoplasmic reticulum (white arrows). (**c**–**h**) Transverse, ultrathin cryosections of smooth-muscle bundles dual-labelled with antibodies against vinculin (vin) and caveolin (cav). Actin-attachment sites (vinculin; **c**, **e**) alternate with caveolar regions (**d**, **e**). Extraction of cholesterol does not affect the actin attachment sites (**f**, **h**) but leads to a redistribution of caveolin (**g**, **h**). Scale bars: (**a**, **b**) 0.5 μm and (**c**–**h**) 5 μm.

RESULTS

Focal adhesions and caveolae-rich zones are spatially separated macrodomains of distinct protein and lipid composition

The smooth-muscle sarcolemma is divided into alternating macrodomains: firm regions of focal adhesions harbouring actin-binding sites and flexible caveolae-rich zones [3,11] (see also Figure 1a).

Each of the membrane macrodomains contains a specific subset of membrane proteins represented in the present study by immunofluorescent labelling of vinculin to delineate the actin-attachment sites (Figures 1c and 1e) and caveolin, outlining vesicular membrane regions (Figures 1d and 1e). To assess if membrane macrodomain organization extends beyond protein constituents and additionally involves the segregation of membrane lipids, we investigated the redistribution of membrane-associated proteins after selective extraction of either cholesterol or glycerophospholipids.

Being a major component of membrane rafts, cholesterol is essential for their stability [17]. Depletion of cholesterol by MCD treatment results in highly specific and localized changes in membrane architecture: whereas plasma membrane integrity is preserved and the sarcoplasmic reticulum remains intact, the caveolae are flattened or disappear completely (Figure 1b). Cholesterol depletion has no effect on the periodic spacing of the focal adhesion sites delineated by vinculin (Figures 1f and 1h). However, it leads to a redistribution of caveolin, which now spreads diffusively around the entire cellular circumference (Figures 1g and 1h).

Constituents of liquid-ordered-raft microdomains are more tightly packed compared with the bulk of the plasma membrane. Consequently, in contrast with non-raft regions, they are resistant to solubilization with non-ionic detergents Triton X-100 and Nonidet P40 at low temperatures [17]. Ultracentrifugation of smooth-muscle microsomes within a discontinuous sucrose gradient containing 1% Triton X-100 throughout, results in the almost complete solubilization of the focal adhesion protein vinculin (Figure 2a), which accumulates together with extracted glycerophospholipids in the 40% sucrose fraction, whereas caveolin 'floats' into the 30% sucrose fraction (Figure 2b) due to its association with cholesterol/sphingomyelin-rich DRMs (detergent-resistant membranes).

Annexins translocate to membrane compartments of different lipid composition

Annexins 2 and 6 preferentially co-localize with the highly buoyant cholesterol/sphingomyelin-rich DRMs (Figures 2c–2e), whereas annexins 1 and 4 are preferentially soluble, co-localizing with extracted glycerophospholipids (Figures 2d and 2e). In contrast with the Ca^{2+}-independent membrane localization of either vinculin or caveolin (results not shown), the annexins' interaction with the plasma membrane is Ca^{2+}-dependent (Figures 2c, 3a–3d).

Depletion of glycerophospholipids with Triton X-100 reduces the sarcolemmal staining of annexin 4 (Figures 3a and 3e), but has a comparatively smaller effect on the membrane association of annexin 6 (Figures 3c and 3g). Presumably, due to unequal penetration of the detergent into the dense smooth-muscle tissue, membrane staining of annexin 4 can be perceived as a gradient, less intense in the left part of the micrograph. Since Triton X-100 treatment leads to membrane perforation, both annexins 4 and 6 are extracted from the cell at low $[Ca^{2+}]_i$ (Figures 3f and 3h).

In contrast, the extraction of cholesterol does not lead to a redistribution of annexin 4 (Figures 3a and 3i), but prevents annexin 6 from associating with the sarcolemma (Figures 3c and 3k). MCD treatment does not perforate the sarcolemma, therefore at low $[Ca^{2+}]_i$, both annexins reside in the cytoplasm (Figures 3j and 3l).

Extraction of membrane-associated proteins by Triton X-100 shows a notable one-by-one extraction pattern

To investigate the compartmentalization of membrane-associated proteins in more detail, we purified and analysed DRMs obtained

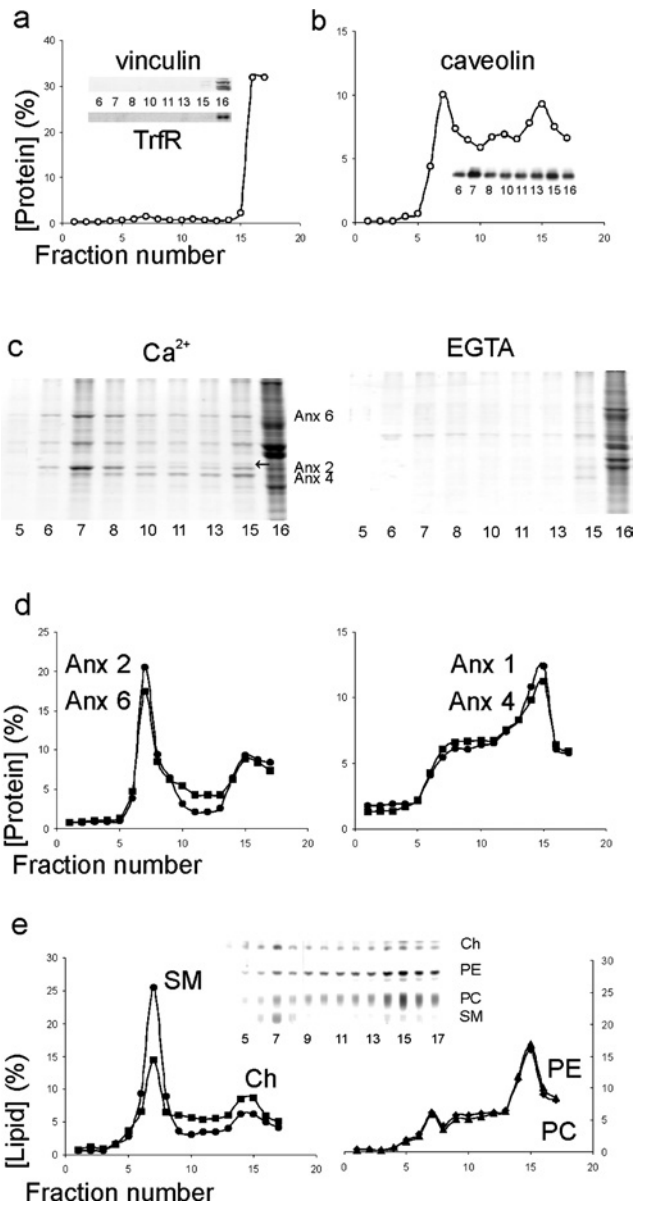

Figure 2 Protein and lipid composition of smooth-muscle DRMs obtained by sucrose-gradient ultracentrifugation in the presence of 1% Triton X-100

The ultracentrifugation was performed in the presence of 200 μM $[Ca^{2+}]$. Fraction 1 corresponds to the top (5% sucrose) of the gradient. Fractions 16 and 17 (40% sucrose) contain solubilized material. (**a**, **b**) Distribution of transferrin receptor, vinculin (**a**) and caveolin (**b**) in the fractions of the sucrose gradient as revealed by Western blotting. (**c**, **d**) Distribution of the annexins as revealed by SDS/PAGE (**c**) and Western blotting (**d**). (**c**) The ultracentrifugation was performed in the presence of either 200 μM $[Ca^{2+}]_{free}$ (Ca^{2+}) or 40 nM $[Ca^{2+}]_{free}$ (EGTA). Position of annexin 1 is marked by an arrow. (**e**) Distribution of cholesterol (Ch), PE, phosphatidylcholine (PC) and sphingomyelin (SM) as revealed by TLC. Anx, annexin.

at various concentrations of Triton X-100. As shown in Figure 4(a), the annexins display a graded extraction pattern (annexin 2 being the least extractable and annexin 1 the most extractable among the four annexins tested) and are more tightly associated with the membranes than vinculin and RhoA, but more easily extracted than caveolin.

To investigate further the membrane compartmentalization, we employed the detergent-extraction method without sucrose gradient ultracentrifugation, as described previously [11]. This method is frequently used in addition to less controllable sucrose

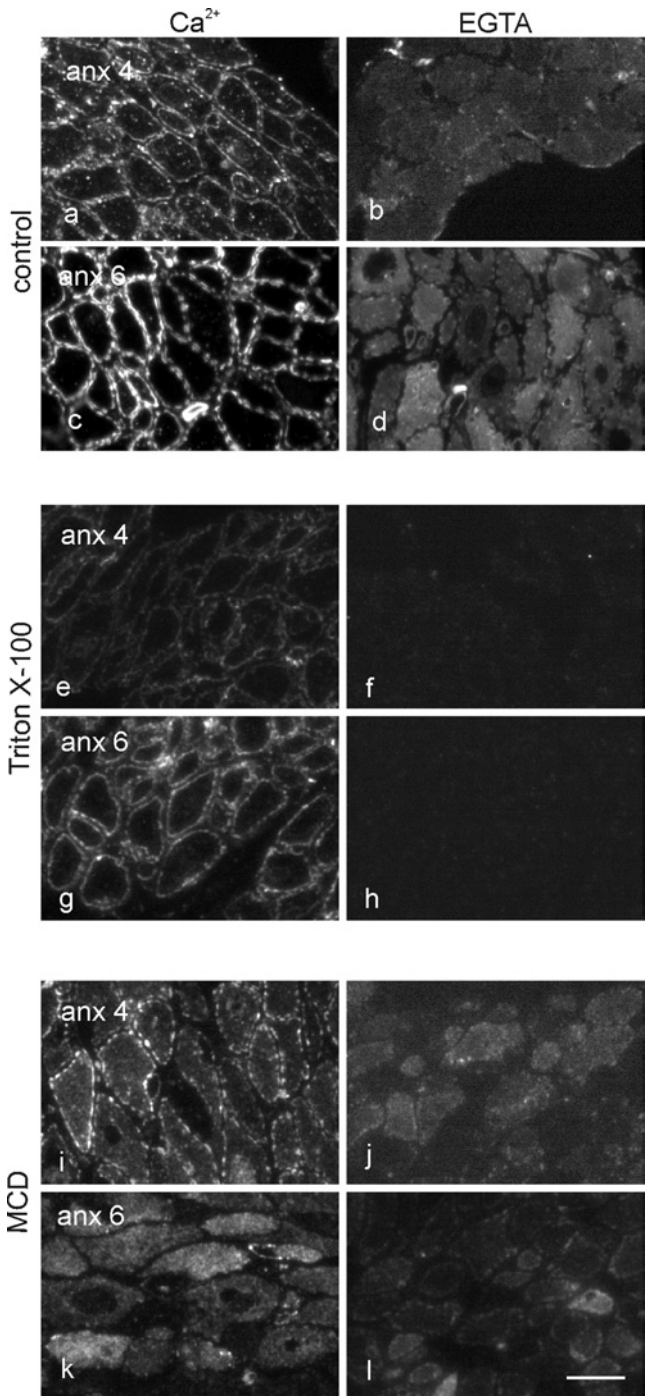

Figure 3 Annexins 4 and 6 associate with distinct lipid partners

Transverse, ultrathin cryosections of smooth-muscle bundles labelled with antibodies against annexin (anx) 4 (**a**, **b**, **e**, **f**, **i**, **j**) or annexin 6 (**c**, **d**, **g**, **h**, **k**, **l**). At high $[Ca^{2+}]_i$, annexins 4 and 6 localize to the sarcolemma (**a**, **c**); after relaxation, both proteins are distributed intracellularly (**b**, **d**) or are completely extracted from the cell by Triton X-100 (**f**, **h**). Treatment with Triton X-100 preferentially removes glycerophospholipids from the membrane and reduces the association of annexin 4 with the membrane at a high $[Ca^{2+}]_i$ (**e**), without affecting the binding of annexin 6 (**g**). Extraction of cholesterol does not affect the localization of annexin 4 (**i**), but prevents membrane association of annexin 6, even at high $[Ca^{2+}]_i$ (**k**). At low $[Ca^{2+}]_i$, both annexins are localized within the cytoplasm (**j**, **l**). Scale bar, 10 μm.

gradient ultracentrifugation to facilitate processing of multiple samples under identical experimental conditions. As shown in Figure 4(b), this method also results in an extraction pattern

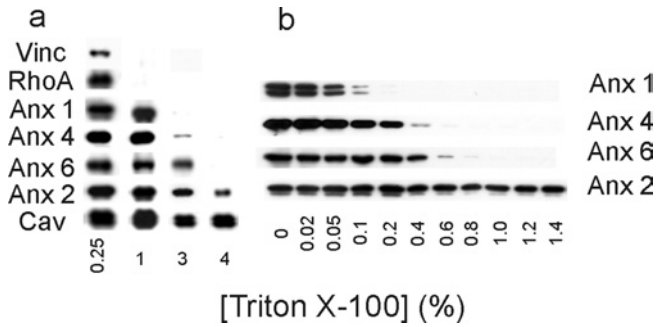

Figure 4 Membrane-associated proteins are selectively and gradually extracted by Triton X-100

(**a**) Protein composition of DRMs obtained by sucrose-gradient ultracentrifugation of smooth-muscle microsomes extracted at 4 °C for 30 min with the indicated [Triton X-100]. (**b**) Smooth-muscle microsomes were incubated at 20 °C for 30 min in the presence of the indicated [Triton X-100]. The suspensions were subjected to low-speed centrifugation. Each of the resulting pellets was analysed by Western blotting. Anx, annexin.

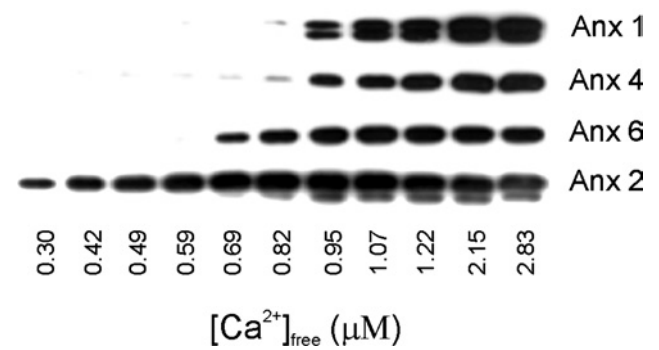

Figure 5 Membrane associations of individual annexins manifest tightly graded Ca^{2+} sensitivities

Smooth-muscle microsomes were incubated at 20 °C for 30 min at the indicated $[Ca^{2+}]_{free}$. The suspensions were subjected to low-speed centrifugation. The resulting pellets were analysed by Western blotting. Anx, annexin.

characterized by gradually increasing extractability: annexin 2 < annexin 6 < annexin 4 < annexin 1. The staggered extraction pattern of individual annexins observed in either experiment indicates that each protein localizes to distinct, probably overlapping lipid microcompartments (Figure 4b).

Annexins act as intracellular Ca^{2+} sensors

In smooth-muscle bundles, the annexins translocate to the plasma membrane in contracted cells, whereas they are diffusely distributed within the cytoplasm of relaxed cells. A map of overlapping Ca^{2+} sensitivities is observed for individual annexins (Figure 5): annexin 2 becomes membrane-associated at a $[Ca^{2+}]_{free}$ of 300 nM, well below the levels for annexin 6 (700 nM) or annexins 4 and 1 ($\sim$1 μM).

Ca^{2+}-dependent extraction of individual membrane lipids

Recently, we have shown that annexin 2 promotes the association of purified DRMs in a Ca^{2+}-dependent manner [11]. To establish if such a Ca^{2+}-dependent association might result in membrane segregation, we simultaneously monitored Triton X-100 extraction profiles of cholesterol and PE (phosphatidylethanolamine) as a function of $[Ca^{2+}]_{free}$ by comparing them with the Ca^{2+} dependence of annexin 2 binding to the plasma membrane. In the presence of 0.4 % Triton X-100, the binding of annexin 2

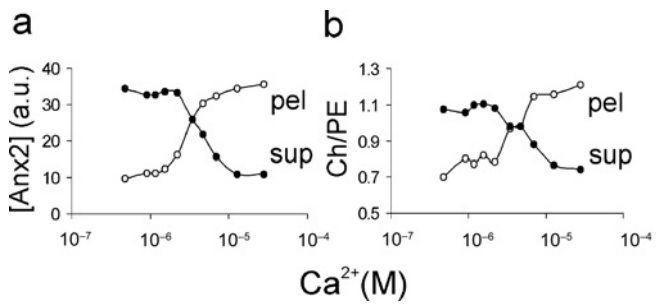

Figure 6 The segregation of membrane lipids is Ca^{2+}-dependent

Smooth-muscle microsomes were incubated at 20 °C for 30 min in the presence of 0.4 % Triton X-100 at the indicated $[Ca^{2+}]_{free}$. The suspensions were subjected to a low-speed centrifugation. Each of the resulting pellets (pel) and supernatants (sup) were analysed by Western blotting for annexin 2 (Anx2) content (**a**) and by TLC for lipid composition (**b**). Note that the Ca^{2+} sensitivity of lipid segregation corresponds to that of annexin 2 binding to the plasma membrane.

occurs at micromolar $[Ca^{2+}]_{free}$ (Figure 6a). Similar to annexin 2 binding, the extraction profile of individual lipids is also Ca^{2+}-dependent: at micromolar $[Ca^{2+}]_{free}$, cholesterol is extracted less efficiently than PE, leading to an increased cholesterol/PE ratio in the resulting detergent-insoluble pellets (Figure 6b). The fact that the Ca^{2+}-sensitivity of changes in cholesterol/PE ratio follows that of annexin 2 binding to the membrane argues for a role of annexin 2 in the stabilization of cholesterol-rich domains.

DISCUSSION

Domain architecture of the smooth-muscle plasma membrane: regulation by $[Ca^{2+}]_i$

Each of the smooth-muscle plasma membrane macrodomains consists of an intricate three-dimensional structure, formed by interactions involving the lipid bilayer, its associated proteins and components of extracellular matrix and intracellular cytoskeleton. Whereas the protein–protein interactions responsible for macrodomain assembly and dynamics have been extensively characterized, the role played by the membrane lipids is much less understood.

Our results suggest that the segregation of structural proteins of the submembrane cortex as well as the segregation of lipid moieties of the lipid bilayer are interdependent in supporting the macrodomain structure of smooth-muscle sarcolemma: caveolae are characterized by a liquid-ordered, cholesterol- and sphingomyelin-rich microenvironment, whereas the focal adhesions preferentially reside in the glycerophospholipid-rich regions (Figure 7).

Most mammalian cells contain two types of cholesterol/sphingomyelin domains; caveolar and non-caveolar rafts [18,19,21]. It is generally taken for granted that both subtypes are similar in their lipid profile yet differ in their spatial organization and protein composition. We propose that caveolin defines stable, Ca^{2+}-insensitive cholesterol/sphingomyelin-rich domains within the smooth-muscle plasma membrane, involved most probably in signalling activities of long duration. In addition, dynamic, Ca^{2+}-regulated cholesterol/sphingomyelin-rich domains might form on the binding of the annexins to the sarcolemma (Figure 7). Annexins 2 and 6 are known to interact with the actin-based cytoskeleton [14]. Thus cholesterol/sphingomyelin-rich domains might be additionally stabilized by annexin-dependent Ca^{2+}-regulated membrane–cytoskeleton complexes [16,20].

Membrane solubilization and thus the lipid and protein composition of DRMs are critically dependent on extraction conditions [21–23]. Even subtle fluctuations such as mechanical handling of the sample during detergent extraction are reported to be of importance [24]. Such a capricious extraction behaviour is incompatible with the predicted 'all' solubilization pattern of proteins associated with that of the bulk membrane, versus the 'nothing' solubilization pattern of proteins associated with the rafts. Therefore the credibility of detergent-extraction methodology has been critically evaluated [23,24]. However, the alternative, microscopic methods used to determine the size and dynamics of lipid rafts gives discouragingly conflicting results (see [12,13] for reviews). Attempts to accommodate contradictory experimental results gave rise to models downgrading lipid rafts to lipid shells [13] or upgrading them to macrorafts [12,25,26].

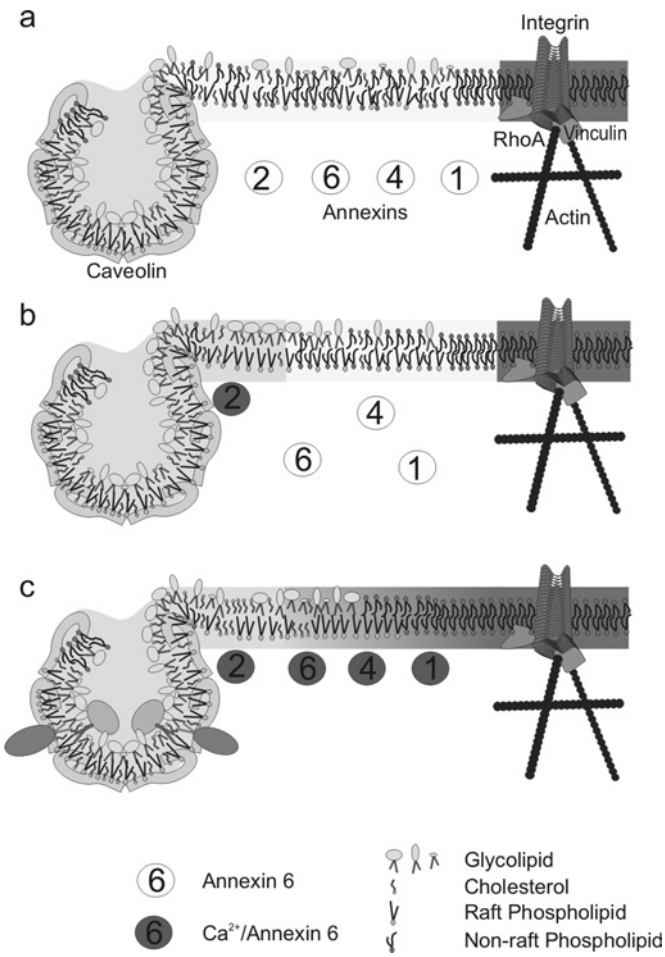

Figure 7 Reorganization of membrane architecture in response to changes in $[Ca^{2+}]_i$

In a smooth-muscle, the periodic linkage of cytoskeletal components to the sarcolemma divides the membrane into preferentially non-raft focal adhesions (integrin, RhoA and vinculin) and into caveolar regions (caveolin). Their dynamics is independent of the cell's contractile state. In addition to the caveolae, smooth-muscle sarcolemma harbours a subset of the highly dynamic non-caveolar rafts. Caveolae and stabilized non-caveolar rafts form together a more extensive lipid superstructure, a macroraft. (**a**) Periodic fluctuations in $[Ca^{2+}]_i$ initiate and terminate each contraction–relaxation cycle. The annexins are localized within the cytoplasm of the resting cells ($[Ca^{2+}]_i$ below 100 nM). Under these conditions, the non-caveolar rafts are present in a non-associated state. (**b**) During the early stages of contraction ($[Ca^{2+}]_i$ below 300 nM), annexin 2 translocates to the sarcolemma and stabilizes the cholesterol-sphingolipid microdomains, thus promoting lipid segregation within a macroraft. (**c**) The increase in $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$ above 600 nM) results in relocation of annexin 6, which assumes its position in the vicinity of the cholesterol-sphingolipid core of the macroraft and is followed (at $[Ca^{2+}]_i$ above 1 μM) by the translocation of annexins 4 and 1 towards the edges of the macroraft. After a decrease in $[Ca^{2+}]_i$, the annexins dissociate from the membrane, leading to destabilization of the macroraft (**a**).

The macroraft models question the existence of permanently separated lipid microdomains and suggest that disordered polyunsaturated glycerophospholipids rather continuously blend into regions dominated by saturated glycerophospholipid species, glycosphingolipids and cholesterol [22,25,27,28]. In contrast with the all-or-nothing raft-extraction profile, the detergent extraction pattern of the macroraft implies differential yet gradual (one-by-one) solubilization of proteins associated within its different compartments. In the present study, we demonstrate that in a smooth muscle, the protein composition of DRMs shows a notable one-by-one extraction pattern. The considerable size of smooth-muscle liquid-ordered domains and the existence of a distinctive substructure within them, demonstrated by single molecule microscopy [9], also support the macroraft concept.

Annexins and membrane segregation: a feedback mechanism for the regulation of Ca^{2+} homoeostasis

Changes in $[Ca^{2+}]_i$ play a central role in the regulation of signalling events. In contractile cells, the activation of receptors and/or changes in membrane potential enhances the sarcolemma's permeability to Ca^{2+} ions and increases $[Ca^{2+}]_i$ (see [1] for a review). The reason why most eukaryotic cells harbour distinct, tissue-specific subsets of Ca^{2+}-sensitive annexins is not immediately obvious [14]. We propose that in smooth-muscle cells, different members of the annexin family work synergistically to form a broad-range Ca^{2+}-sensor: it is the combination of the annexins' graded Ca^{2+} sensitivities that furnishes each cell with a staggering repertoire of responses to fluctuations in $[Ca^{2+}]_i$ (Figure 7).

It has become increasingly evident that the physicochemical properties of a particular microdomain may selectively affect the function of membrane-associated proteins, which are responsible for the maintenance of the Ca^{2+} homoeostasis [29–31]. Our results suggest that the architecture of smooth-muscle plasma membrane might be regulated by $[Ca^{2+}]_i$ through a reversible, annexin-dependent association of lipid microdomains. This rearrangement of plasma membrane compartments might provide a feedback mechanism, which links Ca^{2+} traffic across the sarcolemma with its existing intracellular concentration. Hence the Ca^{2+}-dependent translocation of annexins from the cytoplasm to the sarcolemma can be viewed as part of a system of control loops in which Ca^{2+} modulates the contractile response and self-regulates its own concentration within the cytoplasm.

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